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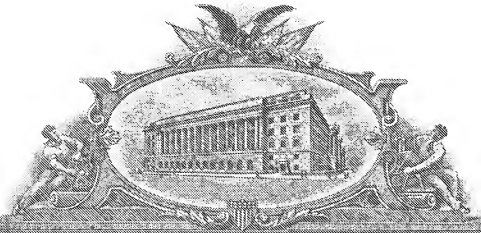

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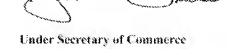

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

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This is a request for filing a Provisional Application for Patent under 37 CFR 1.53(c)

Inventor(s) and Residence(s) (city and either state or foreign country):

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Title: **Sperm Flagellar Energy Carrier Protein as a Contraceptive Target**

25 Sheets of specification.
6 Sheets of sequence listing.
3 Sheets of drawings.

University of Virginia Patent Foundation claims small entity status as a nonprofit organization (37 CFR §§1.27(a)(3) and (c)). The Commissioner is hereby authorized to charge the Small Entity Fee of $80 to Deposit Account No. 50-0423.

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If this invention was made by an agency of the United States Government or under a contract with an agency of the United States Government, the government has certain rights in the invention.

YES ☒ NO ☐ NIH Grant No. TW 00654

Dated: September 30, 2004

Respectfully submitted,

By: Rodney L. Sparks
Rodney L. Sparks (Reg. No. 53,625)

Sperm Flagellar Energy Carrier Protein as a Contraceptive Target

US Government Rights

This invention was made with United States Government support under Grant No. TW 00654, awarded by National Institutes of Health. The United States Government may have certain rights in the invention.

Background

The principal piece is the longest domain of the sperm tail and although lacking mitochondria, it contains major cytoskeletal elements of the flagellum, including axoneme, outer dense fibers and fibrous sheath (FS), the latter structure being restricted to the principal piece. Past reports localized two glycolytic enzymes, hexokinase and GAPDH, to the fibrous sheath, and a role for FS in glycolysis has been posited. However, little is currently known about energy production in the principal piece and there is at present no mechanism to explain energy translocation to dynein ATPases which function as force generating motors along the distal flagella.

Sperm motility depends on a functional flagellum, which consist of several cytoskeletal components including the fibrous sheath. The fibrous sheath is a unique cytoskeletal component in the principal-piece segment of the mammalian sperm flagellum. The fibrous sheath surrounds the axoneme (a motile sliding apparatus) and outer dense fibers and defines the extent of the principal piece region of the sperm flagellum. It consists of two longitudinal columns connected by closely arrayed semicircular ribs that assemble from distal to proximal throughout spermiogenesis. A comprehensive review of the protein composition of the fibrous sheath was recently written (Eddy et al, *Microsc Res Tech.* 2003 May 1;61(1):103-15).

There are several functions for the fibrous sheath that have emerged to date: 1) The fibrous sheath functions as a protective girdle for the sperm axoneme while maintaining flagellar flexibility and affecting the plane of the flagellar beat; 2) The fibrous sheath, through its A kinase anchoring proteins AKAP 3 and AKAP4, serves as a scaffold for enzymes involved in signal transduction including protein kinase A, the Rho

signaling pathway through rhoporrin and rhophilin, and presumably calcium signaling via CABYR; and 3) The fibrous sheath anchors enzymes involved in the glycolytic pathway.

The concept that the fibrous sheath serves as a scaffold for glycolysis is based upon the light and electron microscopic localization of two enzymes of the glycolytic pathway, hexokinase 1 and glyceraldehyde 3 phosphate dehydrogenase to the ribs and longitudinal columns of the fibrous sheath.

There is a need for better means of contraception and a need for rapid, economical, and accurate diagnostic tests for sperm motility and fertility problems. The present invention satisfies these needs.

Summary of Various Embodiments of the Invention

As described herein, additional glycolytic pathway enzymes have now been associated with the fibrous sheath providing further evidence that the fibrous sheath serves as a scaffold for glycolysis. Furthermore, applicants have now discovered that a novel, sperm specific fibrous sheath protein, that is believed to function as an adenine nucleotide translocase, is located in the principal piece of the sperm tail, but not in the midpiece.

The present invention is directed to a sperm flagellar energy carrier protein (SFEC), antibodies specific for SFEC and nucleic acid sequences encoding said protein, as well as compositions comprising such compounds. SFEC is believed to be essential for sperm motility, and thus antagonists of SFEC activity are anticipated to have utility as contraceptive agents. Compositions comprising the amino acid, nucleic acid or antibodies of the present invention can also be used in accordance with the present invention as diagnostic indicators of fertility.

Brief Description of the Drawings

The foregoing summary, as well as the following detailed description of preferred embodiments of the invention, will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings embodiments which are presently preferred. It should be understood, however, that the invention is not limited to the precise arrangements and instrumentalities shown. In the drawings:

Figure 1, comprising Figures 1A, 1B, and 1C, represents an electrophoretic analysis of SFEC protein. Figure 1A is an image of a Coomassie blue stained gel of induced (center lane) and uninduced (right lane) truncated recombinant SFEC (117 amino acid residues) expressed in BLR (DE3) host cells. The arrow indicates the SFEC stained band. The left lane (control) is the molecular weight marker peptide lane. Figure 1B is an image of a Western blot analysis of induced (left lane) and uninduced (right lane) recombinant SFEC using an anti-histidine antibody. Figure 1C depicts an image of affinity purified SFEC (arrow) stained by SYPRO Ruby stain.

Figure 2, comprising Figures 2A, 2B, and 2C, represents a Western blot analysis of recombinant SFEC, human sperm, and isolated FS proteins, using an anti-SFEC antibody. Figure 2A is an image of a Western blot analysis using an antibody against SFEC to detect electrophoresed recombinant SFEC (recSFEC) comparing Post-immune serum (left lane) and Pre-immune serum (right lane). Figure 2B is an image of a Western blot analysis using an antibody against SFEC comparing Post-immune serum (left lane) and Pre-immune serum (right lane) on human sperm. Figure 2C is an image of a Western blot analysis using an antibody against SFEC comparing Post-immune serum (left lane) and Pre-immune serum (right lane) on electrophoresed FS protein.

Figure 3, comprising Figures 3A, 3B, 3C, 3D, 3E, and 3F, represents an indirect immunofluorescence analysis, localizing SFEC to the principal piece of the flagellum of human sperm. Figure 3A represents an image of a phase contrast micrograph corresponding to Figure 3B (FITC only), Figure 3C (FITC + phase), and Figure 3D (FITC + DAPI). Figure 3E is an image of a phase contrast micrograph of sperm. Figure 3F is an image of FITC, corresponding with Figure 3E, of a control experiment using pre-immune serum. Large arrow- principal piece; Small arrow- midpiece.

Detailed Description of Embodiments

Definitions

As used herein, each of the following terms has the meaning associated with it in this section.

The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

"Plurality" means at least two.

As used herein, the term "purified" and like terms relate to an enrichment of a molecule or compound relative to other components normally associated with the molecule or compound in a native environment. The term "purified" does not necessarily indicate that complete purity of the particular molecule has been achieved during the process. A "highly purified" compound as used herein refers to a compound that is greater than 90% pure.

As used herein, the term "pharmaceutically acceptable carrier" includes any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, emulsions such as an oil/water or water/oil emulsion, and various types of wetting agents. The term also encompasses any of the agents approved by a regulatory agency of the US Federal government or listed in the US Pharmacopeia for use in animals, including humans.

A "polylinker" is a nucleic acid sequence that comprises a series of three or more closely spaced restriction endonuclease recognitions sequences.

"Operably linked" refers to a juxtaposition wherein the components are configured so as to perform their usual function. Thus, control sequences or promoters operably linked to a coding sequence are capable of effecting the expression of the coding sequence.

As used herein, "nucleic acid," "DNA," and similar terms also include nucleic acid analogs, i.e. analogs having other than a phosphodiester backbone. For example, the so-called "peptide nucleic acids," which are known in the art and have peptide bonds

instead of phosphodiester bonds in the backbone, are considered within the scope of the present invention.

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

The term "peptide" encompasses a sequence of 3 or more amino acids wherein the amino acids are naturally occurring or synthetic (non-naturally occurring) amino acids. Peptide mimetics include peptides having one or more of the following modifications:

1. peptides wherein one or more of the peptidyl --C(O)NR-- linkages (bonds) have been replaced by a non-peptidyl linkage such as a --CH_2-carbamate linkage (--$CH_2OC(O)NR$--), a phosphonate linkage, a -CH_2-sulfonamide (-CH_2--$S(O)_2NR$--) linkage, a urea (--NHC(O)NH--) linkage, a --CH_2 -secondary amine linkage, or with an alkylated peptidyl linkage (--C(O)NR--) wherein R is C_1-C_4 alkyl;

2. peptides wherein the N-terminus is derivatized to a --NRR_1 group, to a -- NRC(O)R group, to a --NRC(O)OR group, to a --$NRS(O)_2R$ group, to a --NHC(O)NHR group where R and R_1 are hydrogen or C_1-C_4 alkyl with the proviso that R and R_1 are not both hydrogen;

3. peptides wherein the C terminus is derivatized to --C(O)R_2 where R_2 is selected from the group consisting of C_1-C_4 alkoxy, and --NR_3R_4 where R_3 and R_4 are independently selected from the group consisting of hydrogen and C_1-C_4 alkyl.

Naturally occurring amino acid residues in peptides are abbreviated as recommended by the IUPAC-IUB Biochemical Nomenclature Commission as follows: Phenylalanine is Phe or F; Leucine is Leu or L; Isoleucine is Ile or I; Methionine is Met or M; Norleucine is Nle; Valine is Val or V; Serine is Ser or S; Proline is Pro or P; Threonine is Thr or T; Alanine is Ala or A; Tyrosine is Tyr or Y; Histidine is His or H; Glutamine is Gln or Q; Asparagine is Asn or N; Lysine is Lys or K; Aspartic Acid is Asp or D; Glutamic Acid is Glu or E; Cysteine is Cys or C; Tryptophan is Trp or W; Arginine is Arg or R; Glycine is Gly or G, and X is any amino acid. Other naturally occurring amino acids include, by way of example, 4-hydroxyproline, 5-hydroxylysine, and the like.

Synthetic or non-naturally occurring amino acids refer to amino acids which do not naturally occur *in vivo* but which, nevertheless, can be incorporated into the peptide structures described herein. The resulting "synthetic peptide" contains amino acids other than the 20 naturally occurring, genetically encoded amino acids at one, two, or more positions of the peptides. For instance, naphthylalanine can be substituted for trytophan to facilitate synthesis. Other synthetic amino acids that can be substituted into peptides include L-hydroxypropyl, L-3,4-dihydroxyphenylalanyl, alpha-amino acids such as L-alpha-hydroxylysyl and D-alpha-methylalanyl, L-alpha.-methylalanyl, beta.-amino acids, and isoquinolyl. D amino acids and non-naturally occurring synthetic amino acids can also be incorporated into the peptides. Other derivatives include replacement of the naturally occurring side chains of the 20 genetically encoded amino acids (or any L or D amino acid) with other side chains.

As used herein, the term "conservative amino acid substitution" is defined herein as an amino acid exchange within one of the following five groups:

I. Small aliphatic, nonpolar or slightly polar residues:
Ala, Ser, Thr, Pro, Gly;

II. Polar, negatively charged residues and their amides:
Asp, Asn, Glu, Gln;

III. Polar, positively charged residues:
His, Arg, Lys;
IV. Large, aliphatic, nonpolar residues:
Met Leu, Ile, Val, Cys
V. Large, aromatic residues:
Phe, Tyr, Trp

As used herein, "SFEC" represents Sperm Flagellar Energy Carrier Protein. SFEC can be used interchangeably with "testis adenine nuclear transporter" ("tANT").

As used herein, the term "SFEC polypeptide" and like terms refer to a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, and fragments thereof.

As used herein, the term "antibody" refers to a polyclonal or monoclonal antibody or a binding fragment thereof such as Fab, F(ab')2 and Fv fragments.

As used herein, the term "SFEC antibody" refers to an antibody that specifically binds to the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4, or fragments thereof.

As used herein, the term "biologically active fragments" or "bioactive fragment" of an SFEC polypeptide encompasses natural or synthetic portions of the full-length protein that are capable of specific binding to their natural ligand.

The term "non-native promoter" as used herein refers to any promoter that has been operably linked to a coding sequence wherein the coding sequence and the promoter are not naturally associated (i.e. a recombinant promoter/coding sequence construct).

As used herein, a transgenic cell is any cell that comprises a nucleic acid sequence that has been introduced into the cell in a manner that allows expression of a gene encoded by the introduced nucleic acid sequence.

As used herein, the term "treating" includes alleviating the symptoms associated with a specific disorder or condition and/or preventing or eliminating said symptoms. For example, treating cancer includes preventing or slowing the growth and/or division of cancer cells as well as killing cancer cells.

Embodiments

Fertility requires sperm motility and consequently ATP production.

Oxidative phosphorylation in mitochondria is the most efficient way to produce ATP but in the case of spermatozoa, the mitochondria are localized solely in the sperm mid piece and yet the flagella extends another 40 um or so beyond the base of the mid piece. This raises the question of how ATP is generated and made available for the dynein-ATPases of the mitochondrion-free part of the flagellum (principal piece). Accordingly, it has been proposed that glycolysis along the flagellum provides a mechanism for localized ATP production in the principal piece and provides energy for the hyperactivated motility of sperm that allows them to penetrate the zona pellucida. In support of that hypothesis two enzymes of the glycolytic pathway, hexokinase 1 and glyceraldehyde 3 phosphate dehydrogenase have been localized to the fibrous sheath.

The results from one-dimensional SDS-PAGE revealed that the fibrous sheath contains at least 17 distinct Coomassie staining protein bands. These bands were assigned a nomenclature of C253-C269, and each band was cored and microsequenced by tandem mass spectrometry. The results indicated that the isolated fibrous sheath preparation contained many proteins that had been previously characterized as fibrous sheath components including roporrin, AKAP3, AKAP4, GST mu, and GAPDH-2. These findings confirmed the purity of the isolated fibrous sheath preparation. However, more significantly, microsequencing of isolated human fibrous sheath also revealed the presence of five glycolytic proteins, not previously reported to be associated with the fibrous sheath. These enzymes are aldolase A, sorbitol dehydrogenase, lactate dehydrogenase, triosphosphate iosmerase, pyruvate kinase. The addition of 5 new components to the 2 previously known glycolytic enzymes contained in the human fibrous sheath conclusively establishes glycolysis as a process occurring in the principal piece of the sperm flagellum, independent of ATP generation in the mitochondria. Glycolysis is an essential metabolic pathway that may proceed in the absence of oxygen to generate ATP. Accordingly, these findings demonstrate that the fibrous sheath is a flagellar sub-compartment for the glycolytic pathway to generate ATP under anaerobic condition.

Preliminary bioinformatic analysis of the five glycolytic peptides that were obtained from the human fibrous sheath indicated that the glycolytic enzymes represent the somatic form each enzyme, with the exception of the testis specific form of lactate dehydrogenase, LDHC. Although testis isoforms of triose phosphate isomerase have been identified in human (Strausberg et al., Proc. Natl. Acad. Sci. U.S.A. 99 (26), 16899-16903 (2002)), the peptides identified in the fibrous sheath represent the somatic form of TPI rather than the testis isoform. This indicates the fibrous sheath glycolytic machinery is comprised of two subsets of glycolytic enzymes: testis specific as well as somatic isoforms.

The human SFEC protein is 315 amino acids in length, has a molecular weight of 35021.78 daltons, an isoelectric point of 10.4632, a charge of 24.5 and an average residue weight of 111.180.

The nucleotide sequence of the human SFEC mRNA covers 1727 bp including an open reading frame that yields a protein of 315 amino acid residues. The gene structure of SFEC spans approximately 43.8 kb divided into 6 exons and 5 introns. The human SFEC gene was localized to chromosome 4q28.2, while murine SFEC was localized to chromosome 3B. The other known human ADP/ATP carrier proteins in the same family such as heart/skeletal muscle isoformT1 (ANT 1) and liver isoformT2 (ANT 3) were localized to chromosome 4 q35.1 and chromosome X p22.33, respectively. Fibroblast isoform (ANT2) was localized to chromosome X q24. From this evidence indicating the presence of an uncharacterized unique gene the C265 protein is believed to be a novel member of the family of ADP/ATP Carrier Proteins, also known as the ADP/ATP Translocase, or alternatively, Adenine Nuleotide Translocator or ANT. Since the C265 protein was isolated from the fibrous sheath and because a role in signal transduction or glycolysis or both is likely, the novel protein has been designated as a sperm flagellar energy carrier protein or SFEC. At this time it is not yet apparent if SFEC functions as an ATP reserve [storage/sink] or as an ATP carrier which shuttles ATP to the axoneme.

It is known that testis specific isoforms [Hk1-sa, Hk1-sb and Hk1-sc] of hexokinase 1 are produced from a single somatic gene Hk1 [Mori et al 1993] by alternative splicing. In contrast the testis specific form of GAPDH, GAPDS, is encoded by a unique gene locus *Gapds* in mouse and *GAPDH2* in humans. Thus, of the two

known glycolytic enzymes localized in the flagellum, testis specific isoforms exist, and these are generated by either alternative splicing or expression of unique genes. The preliminary bioinformatic analysis of the peptides isolated from the human fibrous sheath indicates that they are all somatic isoforms and do not represent testis specific isoforms, although such forms have been described for triose phosphate isomerase [Strausberg *et al.*, 2002] and LDHC, the germ cell-specific member of the lactate dehydrogenase family [Millan et al., 1987; Strausberg *et al.*, 2002]. This supports the fibrous sheath as being comprised of testis specific and somatic members of the glycolytic enzyme families.

The nucleic acid sequences of human and mouse SFEC are shown as SEQ ID NO:1 and SEQ ID NO:3, respectively and the deduced human and mouse amino acid sequences are shown as SEQ ID NO:2 and SEQ ID NO:4, respectively. The human and mouse SFEC shared 83% identity and 89% similarity of protein sequences.

In accordance with one embodiment of the present invention a purified polypeptide is provided comprising the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4, or an amino acid sequence that differs from SEQ ID NO:2 or SEQ ID NO:4 by 1-5 conservative amino acid substitutions. In one embodiment, the purified polypeptide comprises an amino acid sequence that differs from SEQ ID NO:2 by 20 or less conservative amino acid substitutions, and in another embodiment by 10 or less conservative amino acid substitutions. Alternatively, the polypeptide may comprise an amino acid sequence that differs from SEQ ID NO:2 or SEQ ID NO 4 by 1 to 5 alterations, wherein the alterations are independently selected from a single amino acid deletion, single amino acid insertion and conservative amino acid substitutions. In one embodiment the purified polypeptide comprises the amino acid sequence of SEQ ID NO: 2. The polypeptides of the present invention may include additional amino acid sequences to assist in the stabilization and/or purification of recombinantly produced polypeptides. These additional sequences may include intra- or inter-cellular targeting peptides or various peptide tags known to those skilled in the art. In one embodiment, the purified polypeptide comprises an amino acid of SEQ ID NO: 2 and a peptide tag, wherein the peptide tag is linked to SEQ ID NO: 2. In another embodiment, the purified polypeptide comprises an amino acid of SEQ ID NO: 4 and a peptide tag, wherein the peptide tag is linked to SEQ ID NO: 4. Suitable expression vectors for expressing such

fusion proteins and suitable peptide tags are known to those skilled in the art and commercially available. In one embodiment the tag comprises a His tag.

The present invention also encompasses nucleic acid sequences that encode SFEC. In one embodiment a purified nucleic acid sequence is provided comprising the sequence of SEQ ID NO: 1, SEQ ID NO: 3 or a fragment of SEQ ID NO: 1 or SEQ ID NO: 3. The present invention also encompasses recombinant human SFEC gene constructs. In one embodiment, the recombinant gene construct comprises a non-native promoter operably linked to a nucleic acid sequence comprising SEQ ID NO: 1 or SEQ ID NO: 3. The non-native promoter is preferably a strong constitutive promoter that enables expression of the gene construct in a predetermined host cell. These recombinant gene constructs can be introduced into host cells to produce transgenic cell lines that synthesize the SFEC gene products. Host cells can be selected from a wide variety of eukaryotic and prokaryotic organisms, and two preferred host cells are *E. coli* and yeast cells.

In accordance with one embodiment, a nucleic acid sequence comprising SEQ ID NO: 1 or SEQ ID NO: 3 is inserted into a eukaryotic or prokaryotic expression vector in a manner that operably links the gene sequence to the appropriate regulatory sequences, and SFEC is expressed in the eukaryotic or prokaryotic host cell. In one embodiment the gene construct comprises the nucleic acid sequence of SEQ ID NO: 1 or SEQ ID NO: 3 operably linked to a eukaryotic promoter. Suitable eukaryotic host cells and vectors are known to those skilled in the art. The baculovirus system is also suitable for producing transgenic cells and synthesizing the SFEC genes of the present invention. One aspect of the present invention is directed to transgenic cell lines that express human SFEC and fragments of the human SFEC coding sequence.

In one embodiment the introduced nucleic acid is sufficiently stable in the transgenic cell (i.e. incorporated into the cell's genome, or present in a high copy plasmid) to be passed on to progeny cells. The cells can be propagated *in vitro* using standard cell culture procedure, or in an alternative embodiment, the host cells are eukaryotic cells and are propagated as part of a non-human animal, including for example, a non-human transgenic animal. In one embodiment the transgenic cell is a

human cell propagated *in vitro* and comprises the nucleic acid sequence of SEQ ID NO: 1 or SEQ ID NO: 3.

The present invention also encompasses a method for producing human and mouse SFEC. The method comprises the steps of introducing a nucleic acid sequence comprising a sequence that encodes the human or mouse SFEC into a host cell, and culturing the host cell under conditions that allow for expression of the introduced SFEC gene. In one embodiment the promoter is a conditional or inducible promoter, alternatively the promoter may be a tissue specific or temporal restricted promoter (i.e. operably linked genes are only expressed in a specific tissue or at a specific time). The synthesized SFEC can be purified using standard techniques and used in high throughput screens to identify inhibitors of SFEC activity. Alternatively, in one embodiment the recombinantly produced SFEC polypeptides, or fragments thereof are used to generate antibodies against the human or mouse SFEC. The recombinantly produced SFEC proteins can also be used to obtain crystal structures. Such structures would allow for crystallography analysis that would lead to the design of specific drugs to inhibit SFEC function.

Consistent with SFEC's sequence similarity to other known ADP/ATP carrier proteins and its testis specific expression, the new fibrous sheath protein, SFEC is anticipated to have a function related to the diffusion of ATP (produced by glycolysis) through the principal piece of the flagellum. SFEC may function either as an energy carrier protein for sperm motility or alternatively, as a reservoir of ATP or ADP. Accordingly, this protein represents a target for a small molecule inhibitor that is anticipated to have a contraceptive effect. Such an inhibitor might be effective as either a male contraceptive or an intravaginal spermicidal product.

In accordance with one embodiment of the present invention, a method is provided for isolating agents that inhibit SFEC activity and thus serve as contraceptive agents. More particularly, in one embodiment agents will be screened for their ability to interfere with SFEC's ability to bind ADP and/or ADP. Small molecules that are capable of penetrating the sperm plasma membrane will be highly desirable. In addition the small molecule inhibitors should not be toxic to somatic cells. Isolated SFEC inhibitors will be

used in accordance with the present invention either alone or in conjunction with other contraceptive agents to prevent unintended pregnancies.

In accordance with another embodiment of the present invention an antigenic composition is provided comprising a purified peptide having amino acid sequence SEQ ID NO:2, SEQ ID NO:4, or antigenic fragments thereof. The composition can be combined with a pharmaceutically acceptable carrier or adjuvant and administered to a mammalian species to induce an immune response. Such antigenic compositions have utility for raising antibodies against the SFEC protein for use in diagnostic purposes, or in one embodiment for use in contraceptive vaccine formulations. The vaccines of the invention may be multivalent or univalent. Multivalent vaccines are made from recombinant viruses/vectors that direct the expression of more than one antigen.

Suitable preparations of antigenic compositions include injectables, either as liquid solutions or suspensions; solid forms suitable for solution (or suspension) in liquid prior to injection, may also be prepared. The preparation may also be emulsified, or the polypeptides encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the antigenic composition may also include minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine.

Examples of adjuvants which may be effective, include, but are not limited to: mineral gels, *e.g.*, aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols; polyanions; peptides; oil emulsions; alum, and MDP; N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine, N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine, aluminum hydroxide;.

The polypeptides may be formulated into the compositions as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids, such as, for example, hydrochloric or phosphoric acids, or organic acids such as acetic, oxalic,

tartaric, maleic, and the like. Salts formed with free carboxyl groups may also be derived from inorganic bases, such as, for example, sodium potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

The present invention also encompasses antagonists and agonists, including compounds or nucleotide constructs that inhibit expression or the activity of human SFEC (i.e. transcription factor inhibitors, antisense, interference RNA and ribozyme molecules, antisense oligonucleotides, or gene or regulatory sequence replacement constructs) as well as antibodies that interfere with the activity of SFEC. Antagonists of SFEC activity can be used as contraceptive agents. In accordance with one embodiment a method for identifying antagonists of SFEC activity is provided. The method comprises the steps of contacting an SFEC protein, in the presence and absence of a potential SFEC antagonist, with ATP or ADP or other adenosine derivative and identifying antagonists of SFEC activity based on the ability of said potential SFEC antagonist to decrease binding of ATP or ADP or other adenosine derivative to SFEC. In one embodiment the SFEC protein comprises an amino acid sequence of SEQ ID NO: 2. The present invention also encompasses a method of providing contraception to mammalian species, said method comprising the steps of contacting mammalian sperm cells with a composition comprising an inhibitor of SFEC activity.

In accordance with one embodiment of the present invention an antibody is provided that specifically binds to the human and/or mouse SFEC polypeptide (i.e. SEQ ID NO: 2 or 4). In accordance with one embodiment an antibody is provided that specifically binds to the polypeptide of SEQ ID NO: 2, or to fragments thereof. Antibodies generated in accordance with the present invention may include, but are not limited to, polyclonal, monoclonal, chimeric (i.e "humanized" antibodies), single chain (recombinant), Fab fragments, and fragments produced by a Fab expression library. These antibodies can be used as diagnostic agents for the diagnosis of conditions or diseases characterized by in appropriate expression or overexpression of SFEC (including neoplastic disease), or in assays to monitor the effectiveness of an SFEC agonist, antagonist or inhibitor. The antibodies may be used with or without modification, and may be labeled by joining them, either covalently or non-covalently, with a reporter

molecule. In addition, the antibodies can be formulated with standard carriers and optionally labeled to prepare therapeutic or diagnostic compositions.

Antibodies raised against SFEC can be generated using standard techniques, and include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments, and Fab expression libraries. The antibodies generated can be formulated with standard carriers and optionally labeled to prepare therapeutic or diagnostic compositions. In one embodiment, a composition is provided comprising a SFEC specific antibody and a pharmaceutically acceptable carrier. In one embodiment the composition further comprises a surfactant, adjuvant, excipient or stabilizer. In general, water, saline, aqueous dextrose, and related sugar solution, and glycols such as, propylene glycol or polyethylene glycol, are the liquid carriers, particularly for injectable solutions.

Various procedures known in the art may be used for the production of polyclonal antibodies to SFEC or derivatives or analogs thereof. For the production of antibody, various host animals, including but not limited to rabbits, mice, rats, etc can be immunized by injection with the polypeptide of SEQ ID NO: 2, SEQ ID NO: 4, or a synthetic version, or derivative (*e.g.*, fragment) thereof. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies directed toward the sequence of SEQ ID NO: 2, SEQ ID NO: 4, or fragment thereof, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional

embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). In one embodiment, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for egg surface proteins together with genes from a human antibody molecule of appropriate biological activity can be used; such "humanized" antibodies are within the scope of this invention.

According to the present invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce SFEC single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for SFEC, derivatives, or analogs.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of SFEC, *e.g.*, for imaging these proteins, measuring levels thereof in appropriate physiological samples, in diagnostic methods, etc. The antibodies generated against SFEC antigens can also be used as contraceptive or sterilization agents (i.e. passive immunotherapy), or for use in diagnostic immunoassays or the generation of antiidiotypic antibodies. For example, in one embodiment SFEC antibodies are isolated (*e.g.*, immunoaffinity chromatography, centrifugation, precipitation, etc.) and used in

diagnostic immunoassays, or the antibodies may be used to monitor treatment and/or disease progression. Any immunoassay system known in the art, such as those listed *supra*, may be used for this purpose including but not limited to competitive and noncompetitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme-linked immunosorbent assays), "sandwich" immunoassays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays and immunoelectrophoresis assays.

Another embodiment of the present invention is directed to small molecule inhibitors of SFEC and their use to decrease the motility of mammalian sperm and thus serve as a contraceptive agent. In one embodiment a method of contraception is provided wherein said method comprises the steps of inhibiting the activity of SFEC. Alternatively, the SFEC inhibitory composition may comprise an antisense or interference RNA that prevents or disrupts the expression or activity of SFEC in mammalian sperm cells. In accordance with one embodiment the fertility inhibiting composition comprises one or more active agents selected from the group consisting of small molecule inhibitors, antibodies, antisense RNA and interference nucleic acid sequences.

Interference RNA in mammalian systems requires the presence of short interfering RNA (siRNA), which consists of 19-22nt double-stranded RNA molecules, or shRNA, which consists of 19-29nt palindromic sequences connected by loop sequences. Down regulation of gene expression is achieved in a sequence-specific manner by pairing between homologous siRNA and target RNA. A system for the stable expression of siRNA or shRNA was utilized to generate transgenic animals (Hasuwa et al. FEBS Lett 532, 227-30 (2002), Rubinson et al. Nat Genet 33, 401-6 (2003) and Carmell et al. Nat Struct Biol 10, 91-2 (2003)) and can be used in accordance with the present invention to produce animals whose fertility can be regulated. A conditional interference RNA-based transgenic system would provide the additional benefit of being able to control the level of gene expression at any given stage during the life of the animal. Such a regulatable system would have value in livestock and domesticated animals.

The invention is now described with reference to the following examples. These examples are provided for the purpose of illustration only and the invention should in no way be construed as being limited to these examples but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

In accordance with the present invention, as described above or as discussed in the Examples below, there can be employed conventional clinical, chemical, cellular, histochemical, biochemical, molecular biology, microbiology and recombinant DNA techniques which are known to those of skill in the art. Such techniques are explained fully in the literature.

The invention should not be construed to be limited solely to the assays and methods described herein, but should be construed to include other methods and assays as well. One of skill in the art will know that other assays and methods are available to perform the procedures described herein.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

Examples

Without wishing to be bound by any particular theory, it is hypothesized that the lack of mitochondria in the principal piece and limitations in diffusion of mitochondrial ATP from the sperm midpiece to the distal flagella implies the presence in the principal piece of independent mechanisms for energy production and transport. To identify distal flagellar proteins involved in energy production and transport, a proteomic approach was undertaken to microsequence the insoluble proteins comprising the human fibrous sheath.

Fibrous sheaths were isolated by mechanical and chemical methods, the purity of isolated FS was confirmed by electron microscopy, and the FS proteins were separated by one-dimensional SDS-PAGE, revealing that the fibrous sheath contained at least 17

distinct Coomassie stained protein bands. Each protein band was cored and analyzed by tandem mass spectrometry. Previously known FS proteins were identified including roporrin, AKAP3, AKAP4, GST mu, and GAPDH2. Importantly, five enzymes involved in glucose metabolism and not previously reported to be associated with the fibrous sheath, were identified in the purified preparation. These include aldolase A, triose phosphate isomerase, pyruvate kinase, lactate dehydrogenase C and sorbitol dehydrogenase.

The identification of sorbitol dehydrogenase, a key enzyme in polyol metabolism, strongly suggests a role for this pathway involving the conversion of sorbitol to fructose for use as an energy source in flagellar motility. Bioinformatic analyses of aldolase A, triose phosphate isomerase and pyruvate kinase peptides indicate these enzymes represent somatic forms, whereas purified fibrous sheath contained the testis specific isoform of lactate dehydrogenase, LDHC.

Most strikingly, a novel ADP/ATP translocase, named sperm flagella energy carrier (SFEC), was identified in the fibrous sheath fraction. This previously hypothetical protein was cloned, expressed as a recombinant protein and a specific polyclonal antibody was generated that localized SFEC to the principal piece. This localization established SFEC as a new member of the adenine nucleotide translocase (ANT) family distinct from ANTs that have been isolated from inner mitochondrial membranes. Northern analysis, dot blot analysis, as well as EST databases indicated SFEC is a testis specific ANT.

In sum, this proteomic analysis: 1) expands an understanding of the complement of enzymes involved in energy production and translocation in the principal piece; 2) supports a role for the fibrous sheath in flagellar glycolysis as well as polyol metabolism; 3) indicates that the glycolytic machinery within the principal piece includes somatic as well as testis specific isoenzymes; and 4) provides support for distal flagellar energy metabolism occurring independently from the midpiece mitochondrial sheath. Most importantly, a new hypothesis is advanced that a testis specific ATP/ADP carrier, SFEC, mediates ATP translocation to dynein ATPases involved in sperm motility, defining SFEC as a new contraceptive drug target, and providing a link between energy production and transport in the distal flagella.

GenBank has provided accession number AY550240 for the human SFEC nucleic acid sequence (SEQ ID NO:1).

Methods and Results

Example 1- Expression and Purification of recombinant SFEC protein

A truncated construct (amino acid residues 4-120) of human SFEC (SEQ ID NO:2; 315 amino acid residues) was expressed in bacteria in order to raise a polyclonal antibody. Previous efforts to express the entire SFEC open reading frame were not successful in bacteria, presumably because of the existence of a putative transmembrane domain in the C-terminus. Gene specific primers were designed to create an *Nco*1 site at the 5' end and a *Not*1 site at the 3' end of the polymerase chain reaction (PCR) product according to the human SFEC cDNAs sequences. Primers (Forward primer: 5'-CATGCCATGGAGCCTGCGAAAAAGAAGGCAGAAAAG-3' [SEQ ID NO:5] and Reverse primer: 5'-ATAGTTTAGCGGCCGCCTGTTTTTCTTTATTAACTCCAGA-3' [SEQ ID NO:6]) were obtained from GIBCO BRL (Life Technologies, CA).

SFEC sequences also provided herewith are human nucleic acid (SEQ ID NO:1), human amino acid (SEQ ID NO:2), murine nucleic acid (SEQ ID NO:3) and murine amino acid (SEQ ID NO:4.

PCR was performed with 10 ng of human SFEC cDNAs as a template to obtain the truncated SFEC cDNA using a program of one 2 minutes cycle at 94°C followed by 35 cycles of denaturation, annealing, and elongation at 94°C for 30 second, 50°C for 1 minute and 68°C for 2 minutes. A product of 351 residues in length, which begins at residue position number 129 and ends at residue position number 479 of nucleotide sequences of human SFEC, were separated on a 1% NuSieve (FMC BioProducts, Rockland, ME) agarose gel and sequenced in both directions using vector-derived and insert-specific primers to confirm the sequences.

The cDNA corresponding to the N-terminal 117 amino acids was cloned into the bacterial expression vector pET28b and transformed into *Escherichia coli* strain BLR (DE3) (Novagen, Madison, WI). A single colony was picked from a transformation plate to inoculate 2 liters of LB medium containing 50 μg/ml of Kanamycin and grown at 37°C until the A_{600} reached 0.5. The recombinant protein expression was induced at 37°C for 3

hours with 1mM IPTG (isopropyl-1-thio-β-D-galactopyranoside). The cells were centrifuged at 5,000 g for 15 minutes and suspended in BugBuster Protein Extraction reagent (Novagen, Madison, WI) containing rLysozyme (1 KU/ml) and Benzonase (25 units/ml) for the gentle disruption of the cell wall and degradation of DNA and RNA of the *E. coli*. The cells were centrifuged at 5,000 g for 15 minutes and the pellet of inclusion body was resuspended in 1 X binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) containing 100 μg/ml lysozyme and 8M urea.

The supernatant, urea soluble fraction, was loaded onto a Ni^{2+}-activated His-binding resin (Novagen, Madison, WI) following the manufacturer's protocol. The recombinant protein was eluted with 400 mM imidazole in 1X binding buffer containing 8M urea. The eluted proteins were dialyzed in distilled water, lyophilized at -70°C and performed further purification to a single band using a model 491 Prep Cell (Bio Rad). The purity of the isolated recombinant protein was confirmed by Coomassie and SYPRO Ruby stain (Bio-Rad).

The recombinant SFEC protein containing six residues of histidine on the C-terminus of the protein was induced by 1 mM IPTG (Figure 1A) and confirmed the protein expression using anti-histidine antibody (Figure 1B). The purity of isolated protein of ~13 kDa was verified by SYPRO Ruby stain (Figure 1C).

Example 2- Generation of rat anti-human SFEC antibody

Approximately 100 μg of purified recSFEC protein in PBS emulsified with equal volume of Freund's complete adjuvant was injected subcutaneously and intramuscularly into each female Sprague Dawley rat. Animals were boosted two times at an interval of 21 days with 50 μg of recombinant protein in incomplete Freund's adjuvant and serum was collected 7 days after the second boost. Rats were sacrificed after confirmation of antibody production by Western blot analysis on the recombinant SFEC, human sperm, and isolated FS proteins.

Western analyses of anti-SFEC antibody on the human sperm, isolated FS and recombinant proteins

The reaction of SFEC antibody was tested by Western blot analysis on the recombinant SFEC, human sperm, and isolated FS proteins. The human swim up sperm proteins were extracted as previously described (Shetty *et al.*, *Biol. Reprod.* 61(1):61-9 (1999)). Sperm were solubilized by constant shaking for 2 hours at 4°C in a CELIS lysis buffer containing 9.8 M urea, 2% NP-40, 100 mM DTT and the protease inhibitors: 2 mM PMSF, 5 mM iodoacetamide, 5 mM EDTA, 3 mg/ml L-1-chlor-3-(4-tosylamido)-7-amino-2-heptanon-hydrochloride, 1.46 mM pepstatin A, and 2.1 mM leupeptin. Insoluble material was removed by centrifugation at 10000 x g for 5 minutes, and the supernatant containing solubilized human sperm protein was subjected to one-dimensional electrophoresis.

The proteins resolved by one-dimensional SDS-PAGE were transferred onto nitrocellulose membranes and detected by the anti-SFEC antibody. The excess protein-binding sites on the membrane were blocked with PBS containing 5% (w/v) non-fat milk powder and 0.2 % (w/v) Tween 20 (Merck-Schuchardt, Hohenbrunn, Germany) for 1 hour. The membrane was probed overnight at 4°C with a rat polyclonal antiserum raised against SFEC protein. Anti-SFEC antibody was diluted 1:2000 with blocking solution. Preimmune sera were diluted same as post immune sera for control experiments. The membrane was then incubated for 45 minutes with an anti-rat immunoglobulin IgG-secondary antibody linked to horseradish peroxidase (Jackson Immuno Research Lab., West Grove, PA. USA) diluted 1:5000 in blocking solution. The blot was developed with a chemiluminescent substrate (Pierce, Rockford, IL) or 3,3‘,5,5‘-tetramethylbenzidine (TMB) substrate solution (Kirkegaard and Perry Lab., Gaithersburg, MD. USA).

The positive signal elicited by anti-SFEC antibody was detected intensively on the 38, 32, 20 kDa of human sperm, but only 32 kDa on the isolated FS proteins (Figure 2A). The human sperm and FS proteins were not detected by corresponding preimmune serum (Figure 2B). This result demonstrated that rat anti-human SFEC antibody recognizes SFEC immunogen and human sperm including FS proteins indicating that the SFEC is a component of FS proteins.

Example 3- Localization of SFEC to the principal piece in association with the FS of the flagellum in human sperm

Swim-up human sperm were washed in PBS containing 0.2 mM PMSF, diluted to a concentration of a 1 x 10^6 sperm/ml, and then spotted onto glass slides. The sperm were air-dried, and fixed with 4% paraformaldehyde for 30 minutes at room temperature. After washing 3 times in PBS, the samples were blocked in 10% normal goat serum in PBS overnight at 4°C. The sperm were then incubated with 1:50 dilution of the rat anti-recombinant SFEC antibody or its pre-immune serum in the blocking solution for 2 hours at room temperature. The slides were then washed 3 times x 5 minutes in PBS, and the secondary antibody, goat anti-rat IgG FITC conjugated (Jackson ImmunoResearch), were applied at 1:100 dilution in 10% normal goat serum in PBS for 1 hour at room temperature. The slides were washed 3 times, 5 minutes/wash, in PBS. The Slow Fade-Light Antifade Kit containing DAPI (Molecular Probes, Inc.) was used to stain DNA of the sperm and to reduce the fading rate of the fluorescein.

Indirect immunofluorescence analysis of human swim-up sperm using rat serum against recombinant human SFEC protein localized to the entire principal piece of the flagellum with no staining in the midpiece, endpiece or in the head (Fig. 3 B, C, D). Pre-immune serum showed no immunofluorescence in human sperm (Fig. 3 F) . Interestingly only approximately 50% of sperm were recognized by the SFEC antibody which is directed against N-terminal 117 amino acids. This result demonstrated a previous report that the accessibility of the N-terminal residues depends on the conformational state of the ADP/ATP carrier (Brandolin et al., *Biochemistry* **28**:1093-1100 (1989)). This result indicates that each spermatozoon has different conformation state relating to function for the ADP/ATP translocation in the principal piece of the flagellum.

In summary, the present disclosure provides that SFEC is a novel and unique protein located in the principal piece of the sperm tail and is an appropriate target for a contraceptive drug.

Other methods which were used but not described herein are well known and within the competence of one of ordinary skill in the art of biochemistry, cell biology, and molecular biology.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety. One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention.

Sperm Flagellar Energy Carrier Protein as a Contraceptive Target

Abstract of the Disclosure

The present invention is directed to a sperm flagellar energy carrier, SFEC, antibodies specific for the SFEC and the use of the SFEC protein to identify antagonists of SFEC activity. SFEC is believed to be essential for sperm motility, is localized to a specific region of the sperm, and thus antagonists of SFEC activity are anticipated to have utility as contraceptive agents.

Expression and Purification of recombinant SFEC

FIG. 1A FIG. 1B FIG. 1C

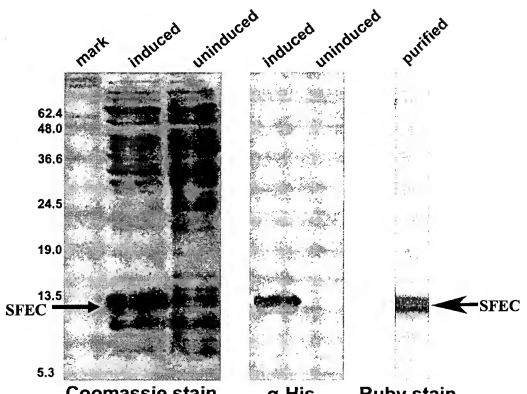

Figure 1. (**A**) Coomassie blue stain of induced and uninduced truncated SFEC (117 amino acids) recombinant protein (arrow) expressed in BLR (DE3) host cell. (**B**) Western analysis of recombinant proteins showing that anti-histidine antibody detects expected molecular weight of ~13 kDa on the induced truncated SFEC protein. (**C**) Affinity purified recombinant SFEC protein (arrow) stained by SYPRO Ruby stain (Bio-Rad) confirming the purity of immunogen before injection into animals.

Western Analysis of SFEC

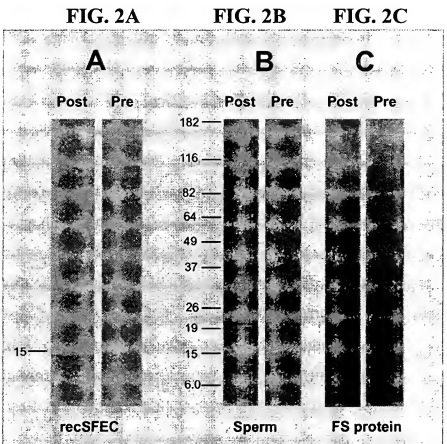

Figure 2. Western blots of anti-SFEC antibody on the recombinant SFEC, human sperm and isolated FS proteins. Post immune serum recognized recombinant SFEC **(A)** and three bands at 38, 32 and 20kDa on the human sperm proteins **(B)**. The FS was recognized at the 32 kDa **(C)** which is initially identified as SFEC from mass spectrometry. Pre immune serum did not recognize any protein of human sperm **(B)**, FS **(C)** or recombinant SFEC **(A)**.

Localization of SFEC to the Principal Piece of the Flagellum

FIG. 3A

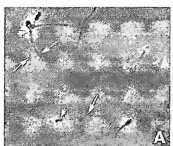

FIG. 3B

FIG. 3C

FIG. 3D

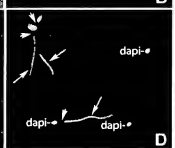

FIG. 3E

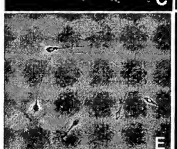

FIG. 3F

Figure 3. Indirect immunofluorescence analysis of human swim-up sperm using rat serum against recombinant human SFEC protein localized to the entire principal piece (large arrow) of the flagellum with no staining in the midpiece (small arrows) or in the head (DAPI). Approximately 50% of sperm were recognized by the SFEC antibody which is directed against N-terminal 120 amino acids.
A, Phase micrograph of corresponding images of **B** (FITC only), **C** (FITC+Phase), and **D** (FITC+DAPI). **E**, Phase micrograph of corresponding image of FITC (F) as a control experiment using pre-immune serum showing no immunofluorescence in human sperm.

SEQUENCE LISTING

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Herr, John
Kim, Young-Hwan

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Leu Gln Val Gln Ala Ser Ser Lys Gln Ile Ser Pro Glu Ala Arg Tyr
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Lys Gly Met Leu Asp Cys Leu Val Arg Ile Pro Arg Glu Gln Gly Phe
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Leu Ser Tyr Trp Arg Gly Asn Leu Ala Asn Val Ile Arg Tyr Phe Pro
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Thr Gln Ala Leu Asn Phe Ala Phe Lys Asp Lys Tyr Lys Glu Leu Phe
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Ile Ala Lys Ser Asp Gly Leu Ile Gly Leu Tyr Gln Gly Phe Gly Val
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Ser Val Gln Gly Ile Ile Val Tyr Arg Ala Ser Tyr Phe Gly Ala Tyr
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Asp Thr Val Lys Gly Leu Leu Pro Lys Pro Lys Glu Thr Pro Phe Leu
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Val Ser Phe Ile Ile Ala Gln Ile Val Thr Thr Cys Ser Gly Ile Leu
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Ser Tyr Pro Phe Asp Thr Val Arg Arg Arg Met Met Met Gln Ser Gly
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Glu Ser Asp Arg Gln Tyr Lys Gly Thr Ile Asp Cys Phe Leu Lys Ile
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Tyr Arg His Glu Gly Val Pro Ala Phe Phe Arg Gly Ala Phe Ser Asn
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Ile Leu Arg Gly Thr Gly Gly Ala Leu Val Leu Val Leu Tyr Asp Lys
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Ile Lys Glu Phe Leu Asn Ile Asp Val Gly Gly Ser Ser Ser Gly Asp
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